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Published in:
Journal of Biological Chemistry

Link to article, DOI:
10.1074/jbc.M806907200

Publication date:
2009

LOCATION OF THE ANTIDEPRESSANT BINDING SITE IN THE SEROTONIN TRANSPORTER: IMPORTANCE OF SER438 IN RECOGNITION OF CITALOPRAM AND TRICYCLIC ANTIDEPRESSANTS.

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Running head: Location of the antidepressant binding site in SERT

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The serotonin transporter (SERT) regulates extracellular levels of serotonin (5-hydroxytryptamine, 5HT) in the brain by transporting 5HT into neurons and glial cells. The human SERT (hSERT) is the primary target for drugs used in the treatment of emotional disorders, including depression. hSERT belongs to the solute carrier 6 (SLC6) family that includes a bacterial leucine transporter, LeuT, for which a high-resolution crystal structure recently has become available. LeuT has proved to be an excellent model for human transporters and has advanced the understanding of SLC6 transporter structure-function relationships. However, the precise structural mechanism by which antidepressants inhibit hSERT and the location of their binding pockets is still elusive. We have identified a residue (Ser438) located within the 5HT-binding pocket in hSERT to be a critical determinant for the potency of several antidepressants including the selective-serotonin reuptake inhibitor citalopram and the tricyclic antidepressants imipramine, clomipramine and amitriptyline. A conservative mutation of Ser438 to a threonine (S438T) selectively increased the K_i for these antidepressants up to 175-fold. The effect of introducing a protein methyl group into the 5HT-binding pocket by S438T were absent or reduced for analogues of these antidepressants lacking a single methyl group. These results suggest that these antidepressants interact directly with the side chain of Ser438 during binding to hSERT; implying an overlapping localization of substrate and inhibitor-binding sites in hSERT suggesting that antidepressants function by a mechanism that involves direct occlusion of the 5HT binding site.

Drugs that inhibit SERT such as the tricyclic antidepressants (TCAs) and the selective serotonin reuptake inhibitors (SSRIs) are widely used to treat emotional disorders such as depression and anxiety (1). Despite the vast clinical significance of SERT as a drug target, structural aspects of drug recognition and inhibition are poorly understood. SERT belongs to the solute carrier 6 (SLC6) family that includes transporters for neurotransmitters such as γ-aminobutyric acid, norepinephrine, dopamine, and glycine. Until recently, the structural mechanism underlying SLC6 transporter function was largely unknown. However, a recent high-resolution crystal structure of a bacterial homolog to mammalian SLC6 transporters, LeuT (2), has provided the first structural insight into SLC6 transporter function and has proven an excellent platform for constructing experimentally validated three-dimensional models of binding pockets

for ions, substrates and inhibitors in the human transporters (3-8).

Similar progress in understanding the structural mechanism of antidepressant inhibition of SERT has so far been absent. Prior to arrival of the LeuT structure, mutagenesis studies have identified several residues at which mutations can perturb potency or binding affinity of SSRIs and TCAs (Fig. 1). LeuT-based homology models of SERT show that several of these residues are localized within the 5HT-binding pocket; thus supporting a model in which antidepressants bind within or in close proximity to the substrate-binding pocket (9). A competitive mechanism is supported by pharmacological studies that show the potency of TCAs and SSRIs at hSERT is sensitive to 5HT concentration (10-12).

However, conclusive interpretation of mutagenesis studies have been hampered by the lack of structural information on hSERT and a consensus regarding the location of the binding sites for TCAs and SSRIs have therefore not been reached. Recently, this lack of consensus was further substantiated when two studies found LeuT to be inhibited by TCAs in a binding pocket outside the substrate-binding site (Fig. 2A) (13,14). Based on mutations of ortholog residues in the human monoamine transporters, Zhou *et al.* (13) proposed that the primary effects of TCAs and possibly SSRIs are mediated by binding in this region in hSERT. Here, we report that a subtle mutation in hSERT, S438T, dramatically affects the potency of antidepressants containing a dimethyl aminopropyl chain. This effect is reduced or absent in analogues of these antidepressants in which a single methyl is removed from the dimethylamino moiety; implicating that antidepressants and Ser438 are in close proximity. Comparative modelling of hSERT suggests Ser438 to be located within the 5HT-binding pocket. In contrast, mutations within the region in hSERT orthologous to the TCA-binding site found in LeuT did not decrease antidepressant potency significantly. Combined with previous data, our results could clarify the current disagreement regarding the position of the binding pocket for antidepressants in hSERT.

Experimental procedures

Chemicals- Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum, trypsin and penicillin–streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). Cell culture flasks and 96-well plates were from NUNC (VWR International, West Chester, PA, USA). [^{3}H]5HT (20.3 Ci/mmol) and MicroScint-20 scintillation cocktail were obtained from PerkinElmer (Waltham, MA, USA). [^{3}H]MADAM (71.0 Ci/mmol) was from GE Healthcare (Buckinghamshire, UK). Imipramine, desipramine, clomipramine, monomethyl-clomipramine, amitriptyline, nortriptyline, citalopram, monomethyl-citalopram, des-methyl-citalopram, (*S*)-citalopram, (*S*)-monomethyl-citalopram, venlafaxine, sertraline, duloxetine, fluoxetine, paroxetine, nisoxetine, MADAM and aminoethyl-citalopram were kindly provided from H. Lundbeck A/S, Copenhagen, Denmark. RTI-55 was purchased from ABX (Radeberg, Germany).

Molecular biology- The mammalian expression plasmid pcDNA3-hSERT containing human SERT cDNA has been described previously (15). Generation of point-mutations (I179D, I179F, D400F, D400K, D400L, L406D, L406F, L406K, S438T, V489D, V489F, V489K, K490D, K490F and K490T) in pcDNA3-hSERT was performed by site-directed mutagenesis using the QuickChange mutagenesis kit (Stratagene, Carlsbad, CA, USA), followed by sequencing of the entire gene (MWG Biotech, Martinsried, Germany).

Cell culturing and expression of human SERTs- COS7 cells (ATCC, Manassas, VA, USA) were cultured in DMEM media with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin at 37 °C in a humidified 5% CO_2 environment. Cells were

transfected using TransIT DNA transfection reagent following the protocol supplied by the manufacturer. Prior to transfection confluent cells growing in monolayer were resuspended in DMEM at a concentration of 1.3 x 10^6 cells/mL. Per 96-well plate, 6 µg DNA and 18 µL transfection reagent were mixed in 0.6 mL DMEM and incubated at 20 °C for 20 min. Subsequently this mixture was added to the cell suspension and immediately afterwards the cells were dispensed into white 96-well plates at 50% confluence.

Uptake assays- Uptake assays were performed +40 h after transfection. Cells were washed twice with PBSCM buffer (in mM: NaCl, 137; KCl, 2.7; Na_2HPO_4, 4.3; KH_2PO_4, 1.4; $CaCl_2$, 0.5; $MgCl_2$, 0.5) prior to uptake experiments. In inhibition studies cells were incubated with increasing concentration of inhibitors in PBSCM and 50 nM or 150 nM [^{3}H]5HT at 20 °C for 30 min. In saturation experiments, cells were incubated at 20 °C for 10 min in PBSCM containing increasing concentrations of [^{3}H]5HT diluted 10-fold with unlabeled 5HT. Uptake was terminated by washing three times with PBSCM. The amount of accumulated [^{3}H]5HT was determined by solubilising cells in scintillant with counting of plates in a Packard TopCounter (Packard Inc., Prospect, CT, USA). Non-specific uptake was determined as uptake in non-transfected cells. Assays were carried out in triplicate and repeated at least 6 times.

Cell membrane preparation and radioligand-binding displacement assay- COS7 cells transiently expressing WT or S438T hSERT growing in 150 mm tissue culture petri plates were washed with PBS with 1 mM EDTA to detach from the plate. Cell suspension was centrifuged at low speed (700 *x* g) at RT for 5 min. Cell pellet was resuspended in cold H_2O and frozen at -20°C for 1 h. The suspension was thawed on ice and subjected to 10-15 passages through a 21 gauge needle to disrupt cells. Homogenate was transferred to cold 2-ml microcentrifuge tubes and centrifuged at 18,000 *x* g at 4 °C for 30 min. Supernatant was aspirated, and the pellet was resuspended in PBSCM. Protein concentration of the resulting membrane preparation was determined according to the BCA method using the Pierce BCA Protein Assay (ThermoFischer, Rockford, IL). Membranes were used directly for binding experiments or stored at -80 °C until use. For saturation binding studies, increasing concentrations of [^{3}H]MADAM and 30-50 µg total membrane protein per sample were combined in 96-well plates and total volume adjusted to 200 µL per sample. Binding was allowed to preceed for 2 h at room temperature with gentle rocking. Subsequently, membranes were transferred to 96-well glass fibre filter plates (Unifilter C, PerkinElmer) preincubated with 0.1% polyethyleneimine using a Packard Bell cell harvester and washed four times with water. Non-specific binding was determined in parallel at membranes from non-transfected COS7 cells. Filter plates were dried and soaked in scintillant followed by counting in a Packard Topcounter (Packard Inc., Prospect, CT, USA). Saturation binding assays were carried out in dublicate and repeated at least five times.

For competition binding assays, 30-50 µg total membrane protein was incubated with a fixed concentration of [^{3}H]MADAM (5 nM for WT and 25 nM for S438T) in the presence of increasing concentrations of inhibitor using the same protocol as for saturation binding experiments. Competition binding assays were carried out in dublicate and repeated at least three times.

Molecular modelling- The crystal structure of LeuT (PDB accession code 2A65) was used to construct a model of hSERT using the MODELLER comparative modelling package (16) following the alignment of SERT and LeuT by Beuming *et al.* (17). The sodium ions were inserted manually in the same position as in 2A65. 5HT was docked into the hSERT model using the Glide docking program (18,19) in Maestro (Schrödinger, LLC, version 8.5) with default settings applied. Since LeuT was crystallized with the

small substrate, leucine, the cavity is rather small for accommodating larger ligands. Therefore, we used a flexible docking approach using the induced fit docking (IFD) procedure in Maestro for docking of (*S*)-citalopram and imipramine. Since the side chain of Phe335 is placed on the extracellular side of the binding site as an aromatic lid, this side chain prevents access into the cavity. Therefore, the option in the IFD workflow, in which this residue initially is mutated to an Ala residue and then added again later in the refinement, was used.

Data analysis- All data analysis was performed using Prism 4.0 software (GraphPad Inc, San Diego, CA, USA). For determination of IC_{50} values dose-response data from [^{3}H]5HT uptake inhibition assays were fitted by the equation:

$$\% \text{ specific uptake} = 100/(1 + 10\text{^}\{(\log IC_{50} - \log[\text{inhibitor}]) \times \text{Hill slope}\})$$

, where IC_{50} is the concentration of inhibitor that produces a half-maximal inhibition of uptake. For determination of K_m (the Michaelis-Menten constant) and V_{max} (maximal uptake rate) the uptake rate was plotted as function of substrate concentration and fitted by the equation:

$$\text{Uptake rate} = (V_{max} \times [5HT])/(K_m + [5HT])$$

IC_{50} values were converted to K_i values using the Cheng-Prusoff equation (20):

$$K_i = IC_{50}/(1+([L]/K_m))$$

, where [L] is the concentration of [^{3}H]5-HT. K_i values were compared using Student's *t*-test unless otherwise indicated.

For determination of K_d (the dissociation constant) data from saturation binding experiments were fitted to the equation:

$$\text{Specific binding} = (B_{max} \times [\text{ligand}])/(K_d + [\text{ligand}])$$

, where B_{max} is the maximal binding, and K_d is the concentration of ligand required to reach half-maximal binding. For determination of IC_{50}, dose response data from competition binding experiments were fitted to the equation:

$$\text{Specific binding} = 100/(1 + 10\text{^}\{\log[\text{inhibitor}] - \log IC_{50}\})$$

, where IC_{50} is the concentration of inhibitor that produces a half-maximal binding of radioligand. IC_{50} values were converted to K_i values using the Cheng-Prusoff equation:

$$K_i = IC_{50}/(1+([L]/K_d))$$

, where [L] is the concentration of [^{3}H]MADAM.

RESULTS

The conservative mutation S438T selectively affects hSERT inhibitors. The importance of Ser438 for antidepressant binding was initially discovered during a LeuT-guided mutational study of hSERT aiming at identifying residues forming the binding-pocket for the SSRI citalopram (Andersen *et al.*, Soc. Neurosci. Abstr. 248.15, 2007). Our initial observation was that a conservative mutation of Ser438 to a threonine (S438T), thus introducing only a methyl group, increased the K_i for citalopram inhibition of [^{3}H]5HT uptake 175-fold (Fig. 2C-D) (P<0.0001; N=6, paired t test). The K_m value for 5HT transport was 6.9-fold decreased by this mutation (0.97 ± 0.09 µM, N=9, for WT vs. 0.14 ± 0.02 µM, N=4, for S438T, P<0.0001, t test), and V_{max} was 5.5-fold decreased (6.6 ± 1.1 pmol/well/minute, N=9, for WT vs. 1.2 ± 0.1 pmol/well/minute, N=4, for S438T; P<0.0001, t test). Concomitant characterization of 11 prototypical hSERT inhibitors showed a remarkably selective influence of S438T on inhibitor potency (Fig. 2C; Table 1; supplementary Fig. S1; supplementary Table

S1). Specifically, seven inhibitors showed decreased K_i at S438T compared to WT ranging from 7-fold for the TCA imipramine to 450-fold for the cocaine analog RTI-55. The remaining compounds displayed no or less than a 2-fold change in K_i.

Ser438 is located in the 5HT-binding pocket. Structural sequence alignment show that Ser438 in hSERT corresponds to Ser355 in LeuT (Fig. 2B)(2,17,21). In LeuT, Ser355 is located in the substrate-binding pocket interacting directly with the alkyl side chain of the substrate leucine and a Na^+ ion. Previous experiments with hSERT have identified several residues important for substrate recognition. Most of these residues align well with residues in LeuT that form the substrate-binding pocket (21); substantiating that LeuT is a valid structural model of the 5HT-binding pocket in hSERT. In particular, evidence for a key role of Ser438 in 5HT recognition has recently been shown in hSERT (8). In LeuT, Ser355 is located 5 Å from Gly24, which is replaced by an aspartate in hSERT (Fig. 2B; Asp98 in hSERT) (2). This aspartate residue is conserved in all SLC6 transporters that transport monoamines (5HT, dopamine, norepinephrine), and several lines of experimental data have established that the aspartate carboxylate group coordinates the primary amino group of monoamines (7,8,22-24). All TCAs and SSRIs contain an essential amino group that has been proposed to form similar interactions with Asp98 in hSERT (23). In our LeuT-based homology model of hSERT shows that Ser438 is located within 4 Å of Asp98 (Fig. 2B). Considering this close proximity of Asp98 to Ser438 in hSERT, the S438T mutation might thus specifically perturb the ability of the substrate-binding pocket to accommodate the aminopropyl chain of citalopram.

Reciprocal modification of SSRIs and TCAs revoke the effect of the S438T mutation. We hypothesized that a citalopram analogue with a shorter aminoalkyl chain would be less affected by the S438T mutation. We tested this idea by evaluating the effect of S438T on a citalopram analog containing a shorter aminoalkyl chain and we found no difference in K_i between WT and S438T hSERT (Fig. 2D; supplementary Table S1) (P=0.11, N=6; t-test). This striking effect indicates that S438T may introduce a steric clash between the dimethyl aminopropyl chain of citalopram and the γ-methyl group of the threonine (Fig. 3). To further explore this, we determined the effect of S438T on citalopram analogs where one or two methyl groups were removed from the aminopropyl chain; anticipating these subtle modifications to gradually decrease the effect of the S438T mutation on inhibitor potency. Indeed, K_i for the monomethyl and des-methyl citalopram analogs (Fig. 4; Table 1) were remarkably less affected by the S438T mutation displaying only a 5.8-fold and 1.7-fold loss in potency, respectively, compared to the 175-fold decrease observed for citalopram (P<0.0001, N=6 [citalopram vs. monomethyl-citalopram]; P<0.0001, N=6 [monomethyl-citalopram vs. des-methyl-citalopram]; t test). This demonstrates that the addition of the protein methyl group at position 438 is compensated by removal of methyl groups on the aminopropyl chain of the ligand. We also characterized (*S*)-citalopram, which is the more potent enantiomer of the racemic citalopram, and its monomethyl analogue, (*S*)-monomethyl-citalopram at WT and S438T and observed a similar pattern with 320-fold loss of potency for (*S*)-citalopram compared to only a 12-fold loss of potency for (*S*)-monomethyl-citalopram (P=0.0003, N=6-8 [(*S*)-citalopram vs. (*S*)-monomethyl-citalopram]; t test) (Table 1).

The ability of the S438T mutation to differentiate between one or two methyl groups on the aminopropyl chain of citalopram indicates that Ser438 is a direct contact site for citalopram. In order to investigate the generality of this phenomenon, we investigated the effect of S438T on another major class of antidepressants, the TCAs. The TCA imipramine contains the

same dimethyl aminopropyl chain as citalopram, and desipramine is the monomethyl analogue of imipramine. This close analogy to citalopram and monomethyl-citalopram allowed us to test if the potency at hSERT of these TCAs also is differentially affected by the S438T mutation following similar patterns. We therefore determined the K_i for imipramine and desipramine at WT and S438T (Fig. 4; Table 1). Indeed, we observed a similar pattern where the S438T mutation induced a 7.1-fold loss of potency for the dimethyl analogue imipramine in contrast to a much smaller 1.7-fold loss for the monomethyl analogue desipramine (P<0.0001, N=6 [imipramine vs. desipramine]; *t* test).

Next, we characterized clomipramine and its monomethyl analog, monomethyl-clomipramine, at WT and S438T and observed the same pattern (Fig. 4; Table 1); clomipramine having an 11.4-fold loss of potency whereas monomethyl-clomipramine displayed only a 4.0-fold loss (P=0.0084, N=8 [clomipramine vs. monomethyl-clomipramine]; *t* test). Finally, we characterized the TCAs amitriptyline and nortriptyline, differing in the same way as the other TCAs (Fig. 4; Table 1). Amitriptyline showed an 11.5-fold loss of potency whereas nortriptyline had no change of potency on S438T compared to WT (P<0.0001, N=8 [amitriptyline vs. nortriptyline]; *t* test).

Competitive binding analysis of the S438T mutation. Rather than directly perturbing ligand interaction with hSERT, the effects of the S438T mutation on the inhibitory potency of antidepressants could be caused via long range allosteric modification of the inhibitor-binding pocket. Therefore, we performed [^{3}H]MADAM competitive binding assays to test if S438T-induced changes in inhibitory potency reflect a concomitant loss in binding affinity of the inhibitors (Table 2). [^{3}H]MADAM was used to label SERT since the apparent K_i-value of this radioligand was almost insensitive to the S438T mutation in contrast to other hSERT radioligands ([^{3}H]imipramine, [^{3}H]paroxetine, [^{3}H]escitalopram, [^{125}I]RTI-55). Saturation binding analysis of [^{3}H]MADAM showed that the K_d-value was increased at hSERT S438T compared to hSERT WT (1.3 ± 0.3 nM, N=6, for WT vs. 21.6 ± 3.7 nM, N=5, for S438T). Results from the competition binding assays showed that the effects of the S438T mutation on inhibitor binding follow the same trends as observed in the functional uptake inhibition studies (Table 2), i.e. the affinity decrease caused by introduction of the protein methyl group in the S438T mutant was significantly smaller for all inhibitors containing a monomethyl aminopropyl chain than the affinity decrease observed for their dimethyl congeners. Notably, when we compare data from competition binding assays to the data obtained from the functional 5HT uptake inhibition assays, we observed that the loss of binding affinity was greatly amplified for inhibitors having a dimethyl propylamino chain compared to the loss in potency. For instance, citalopram showed a 175-fold loss of potency at the S438T mutant while the binding affinity was decreased by 2148-fold. The same dramatic effect of S438T on binding affinity compared to potency was observed for the dimethyl analogues imipramine (283- vs. 7-fold) and clomipramine (107- vs. 11-fold). In contrast, the impact of S438T on binding affinity of the monomethyl analogues was found to be in the same range as that observed in the functional assay (3- to 11-fold vs. 1- to 6-fold). Thus, data from the competition binding assays show that the S438T mutation indeed has a even more pronounced effect on the ability of hSERT to bind inhibitors with a dimethyl propylamine chain compared to their monomethyl congeners.

Induced fit docking of citalopram and imipramine. The inhibitory potency of hSERT inhibitors containing a dimethyl aminopropyl moiety are more sensitive to the S438T mutation compared to their monomethyl congeners. However, citalopram is remarkable more affected by the mutation compared to the three TCA molecules having

a dimethyl aminopropyl chain (imipramine, clomipramine and amitriptyline). To better understand these differences, we performed induced fit docking of (*S*)-citalopram and the prototypical TCA, imipramine, into our homology model of hSERT (Fig. 5; supplementary Fig. S2). The dimethyl amino group of both inhibitors are stabilized by the carboxylate group of Asp98. However, the dimethyl amino group of (*S*)-citalopram is located in closer proximity of Ser438 compared to the same moiety of imipramine (4Å vs. 6Å). Since the hydroxy group on the side chain of Ser438 also coordinates one of the sodium ions, it cannot interact with the antidepressants through a hydrogen-bond, but is important in defining the cavity around the amino group. Given the difference in proximity of the inhibitors and Ser438, we suggest that citalopram would be more sensitive to the introduction of a methyl group at Ser438 compared to imipramine. Since imipramine is located further away from Ser438 compared to (*S*)-citalopram, the steric clash between a methyl group on the dimethyl aminopropyl chain and the introduced protein methyl group at S438T would affect imipramine to a lesser extent than (*S*)-citalopram. This idea is supported by the experimental results showing TCAs to be less affected by the S438T mutation compared to citalopram.

Role of hSERT vestibule residues for antidepressant binding. The ability of S438T to discern the presence of methyl groups on antidepressants suggest that this residue is a direct contact point for these ligands; implicating the 5HT-binding pocket as a likely binding site for these inhibitors. In contrast, the TCA-binding pocket recently found in LeuT is located in a region that forms a vestibule in the substrate permeation pathway that is probably shared by all SLC6 transporters (13,14,25,26). The orthologous region in hSERT could presumably form a secondary binding pocket for antidepressants and contribute to the inhibitory mechanism; by influencing the *en route* passage to the primary binding pocket or by allosteric modulating binding at the primary pocket. We therefore examined the contribution of this region in hSERT to antidepressant function by introducing a range of non-conservative mutations (Fig. 6; supplementary Table S2). Specifically, we introduced considerably different side chains at five positions in order to perturb potential molecular contact to the antidepressants. Of the resulting 14 mutants, 9 had intact 5HT transport function, but none of these mutations increased K_i values significantly for (*S*)-citalopram, imipramine, or clomipramine. In contrast, a few mutants displayed slightly decreased K_i. Based on this lack of effect of vestibule mutations to perturb antidepressant potency, we suggest that this region in hSERT has minor influence on the inhibitory mechanism of TCAs and SSRIs.

DISCUSSION

Fundamental requirements for understanding the structural mechanism underlying antidepressant inhibition of hSERT include unambiguous identification of the location of inhibitor-binding site and elucidation of specific protein-ligand contacts. The LeuT structure has great potential to guide functional studies aiming at providing this information, but in order to take full advantage of this structural template, it is critically important to establish similarities and discrepancies between the bacterial transporter and its mammalian relatives. Zhou *et al.* (13) suggests TCAs and possibly SSRIs inhibit human monoamine transporters by the same mechanism as observed in LeuT; implicating that the primary binding site is located in the transporter vestibule, separated from the substrate-binding pocket. This hypothesis seems in conflict with the fact that SSRIs and TCAs act as competitive inhibitors at hSERT (10-12). However, accessibility of a distinct binding pocket in the vestibule is likely to vary during transport activity and could therefore be sensitive to increasing 5HT concentrations. Furthermore, recent structural

simulations of LeuT have shown that the vestibule in LeuT holds a second substrate binding site (27). Thus, the vestibule in SERT might also have a second 5HT-binding site that is occupied during substrate permeation towards the central binding pocket.

However, comprehensive mutational studies have identified residues in the 5HT-binding pocket that are critical determinants for recognition of inhibitors (Fig. 1). In particular, Henry *et al.* (28) found that mutation of Ile172 on TM3 decreased potency of the SSRIs citalopram and fluoxetine by up to 2 orders of magnitude. Ile172 likely corresponds to Val104 in LeuT, which is located in the leucine binding pocket (2). Celik *et al.* (8) have recently verified that Ile172 has an ortholog position in the substrate binding site in SERT. TM3 is the antiparallel homolog of TM8 and it is noteworthy that Ser438 and Ile172 are located on opposite sides of the 5HT-binding pocket. Together these data are best explained by an overlapping binding site for 5HT and inhibitors in which Ile172 and Ser438 form direct contact points for inhibitor binding. However, differentiation between direct and indirect effects in mutagenesis studies is inherently difficult. Effects from a mutation could arise from a long-range allosteric effect that perturbs an inhibitor-binding site physically distinct from the 5HT pocket. A mutation could also induce a shift in equilibrium between the conformational states SERT assumes during substrate translocation. In both cases, the temporal accessibility of the inhibitor-binding site decreases. Indeed, it has been observed for imipramine that the dissociation rate is decreased in the presence of 5HT, which suggest cooperativity between two physically distinct binding sites (29). Therefore, the effects of mutation of Ile172 and Ser438 cannot be interpreted unambiguously without substantiating direct interactions between these residues and the inhibitor molecule. In the present study, we address this problem by showing that analogs of TCAs and SSRIs with reciprocal chemical modifications become insensitive to the S438T mutation; substantiating a steric clash between the inhibitor aminopropyl moiety and the introduced protein methyl group as reason for the observed loss of potency. Similar approaches have previously been used to map specific interactions between 5HT-binding pocket residues and functional 5HT groups (8,22,23). This implies that the Ser438 side chain is within 3 Å of the ligand and likely constitutes a direct contact point for the aminopropyl group of these inhibitors. In comparison, the distance between the equivalent residue in LeuT, Ser355, and the TCA molecule within the structure of LeuT in complex with TCA is 18 Å (PDB code 2QJU); thus suggesting the antidepressant-binding pocket in LeuT and hSERT to be separate structural entities and supporting that 5HT- and antidepressant-binding pockets overlaps in hSERT.

While the I172M and S438T mutants specifically indicate that the location of the hSERT high-affinity binding pocket for citalopram and several TCAs is different from the LeuT TCA-binding pocket, the orthologous region in hSERT could still hold a secondary inhibitor-binding site. There is substantial evidence for the existence of a secondary low-affinity allosteric site that can modulate dissociation rates of several hSERT ligands from the high-affinity binding site (29). Although further experiments are needed to fully address the existence of functional relevant vestibule inhibitor-binding pocket, our observation that non-conservative mutations in this region in hSERT fail to alter inhibitor K_i indicate that, if present, such a binding site has little influence on the overall inhibitory mechanism.

The results presented imply Ser438 as a direct contact for antidepressants bearing an aminopropyl group and provide further evidence for the location of a high-affinity antidepressant pocket in hSERT. Along with other SERT mutants, S438T is potentially useful for future studies of the structure of

this binding pocket and the mechanism of inhibition of antidepressants.

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FOOTNOTES

* This work was supported by the Carlsberg Foundation and the Danish Medical Research Council (SSVF) (L.O.), the Lundbeck Foundation (K.B.H.), the Danish Council for Strategic Research NABIIT programme (O.T.), and the Drug Research Academy (Ph.D. stipend to J.A.). We thank Dr. Benny Bang-Andersen for providing compounds from the H. Lundbeck A/S compound collection and Prof. Flemming S. Jørgensen for fruitful discussions and assistance with computational studies. We thank Drs. Claus J. Løland and Kevin Erreger for critical comments on the manuscript.

The abbreviations used are: 5HT, 5-hydroxytryptamine; hSERT, human serotonin transporter; LeuT, leucine transporter; SSRI, selective serotonin reuptake inhibitor; SLC6, solute carrier 6; TCA, tricyclic antidepressants.

FIGURE LEGENDS

Fig. 1. Topology of the human serotonin transporter. The substrate-binding pocket is formed by residues positioned in TM1, TM3, TM6 and TM8 (shown in grey). Residues elucidated as critical in recognition of SSRIs and TCAs are indicated: Tyr95 (30), Asp98 (23), Ala169 (31), Ile172 (28), Met180 (32), Ser276 (33), Phe513 (32), Ser545 (34) and Phe586 (35).

Fig. 2. Mutation of Ser438 in the hSERT substrate binding pocket affects the inhibitory potency of antidepressants. *A*. Structure of LeuT in complex with leucine and desipramine (PDB code 2QJU). TM1 (*blue*), TM8 (*green*), and TM10 (*pink*) are highlighted. TM6 and TM11 are removed for clarity. Leucine and desipramine are shown as CPK representations in yellow and orange, respectively. Arg30 and Asp404 (shown as sticks) form the extracellular gate that separates leucine from the bound desipramine. *B*. *Left*: Substrate binding site in LeuT (PDB code 2A65). *Right*: Homology model of the substrate binding site in hSERT. Conserved residues are shown in grey and divergent residues are shown in dark-red. TM1 (*blue*), TM6 (*grey*) and TM8 (*green*) are shown in both panels. *C*. Graphical summary of the fold change (mean ± S.E.M.; N=6-8) in potency of inhibitors at hSERT S438T compared to hSERT WT. K_i values and statistics are shown in Table 1 and supplementary Table S1. # indicates that fold change is less than 1. *D*. Inhibition of 5HT uptake at hSERT WT (■) and S438T (□) by citalopram and aminoethyl citalopram. The shown concentration-response curve is a composite of six independent experiments. Error bars are S.E.M. and are shown when larger than symbol size.

Fig. 3. Proposed working model for the impact of S438T mutation. Cartoon showing hSERT interaction with the aminopropyl chain of TCAs and SSRIs. Asp98 on TM1 (*blue*) coordinates the

aminopropyl group. Introduction of a methyl group at Ser438 on TM8 (*green*) by the S438T mutation leads to steric clash (indicated by the red stipulated line) with a methyl group on the aminopropyl chain. The steric clash is removed with monomethyl analogs.

Fig. 4. Functional [^{3}H]5HT uptake inhibition uptake analysis at hSERT WT and hSERT S438T. *A. Upper:* Structure of inhibitors having one (R = H) or two (R = CH_3) methyl groups at the aminopropyl chain. *Middle:* Characterization of pairs of inhibitors having a single (■[WT]; ▲[S438T]) or two (■[WT]; ▲[S438T]) methyl groups at the aminopropyl chain. *Lower:* Panel summarizes observed fold-change in K_i between WT and S438T for each inhibitor pair (mean ± S.E.M.; N=6-8). K_i values and statistics are shown in Table 1.

Fig. 5. Induced fit docking of (*S*)-citalopram (*A*) and imipramine (*B*). The highest scoring binding modes of both compounds are shown. TM1 (*blue*), TM3 and TM8 (*green*) are shown in both panels. Distances between the amine groups of the inhibitors and the carboxylate oxygen atom of Asp98 and the Cβ-atom of Ser438 are shown.

Fig. 6. Impact of vestibule mutations in hSERT on inhibitor potency. *A. Left*: Structure of the TCA binding site in LeuT with imipramine bound (PDB code 2Q72). *Right*: Overlay of homology model of hSERT with LeuT structure (shown in left panel) to show the equivalent site in hSERT. TM1 (*blue*), TM3, TM6, EL4 and TM10 (*pink*) are shown. *B*. Graphical summary of fold change (mean ± S.E.M.; N=6-10) in potency of (*S*)-citalopram, imipramine and clomipramine at hSERT-mutants compared to hSERT-WT. For comparison, the fold change in potency for (*S*)-citalopram, imipramine and clomipramine at S438T compared to hSERT WT are shown. K_i values and statistics are shown in supplementary Table S2. * indicates a significant change ($P < 0.05$) in K_i values.

Table 1. Impact of hSERT S438T on [^{3}H]5HT uptake inhibition constants for SERT inhibitors

The K_i-values for SERT inhibitors were determined at hSERT WT and hSERT S438T in a [^{3}H]5HT uptake inhibition assay as described in "Experimental Procedures". Data represents mean ± S.E.M. from at least 6 independent experiments (number of replicates given in parantheses) each performed in triplicate. The number of methyl-groups on the aminopropyl chain of the inhibitors is indicated. **, $P < 0.01$; ***, $P < 0.001$ (Student's t test).

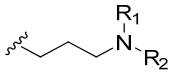
Compound			hSERT WT K_i (nM)		hSERT S438T K_i (nM)		Affinity change	
	R_1	R_2					K_i(S438T)/K_i(WT)	
Imipramine	CH_3	CH_3	153.9 ± 21.7	(6)	1070.6 ± 102.3	(6)	7.1 ± 0.3	***
Desipramine	CH_3	H	1515.3 ± 173.7	(6)	2639.3 ± 351.2	(6)	1.7 ± 0.1	
Clomipramine	CH_3	CH_3	101.5 ± 32.7	(8)	717.9 ± 92.7	(8)	11.4 ± 2.4	**
Monomethyl-clomipramine	CH_3	H	589.5 ± 91.7	(8)	2294.9 ± 267.1	(8)	4.0 ± 0.2	
Amitriptyline	CH_3	CH_3	634.7 ± 149.0	(8)	6044.8 ± 733.9	(8)	11.5 ± 1.4	***
Nortriptyline	CH_3	H	3158.0 ± 587.8	(8)	2693.3 ± 334.4	(8)	0.9 ± 0.1	
Citalopram	CH_3	CH_3	59.3 ± 6.7	(6)	10038.3 ± 530.6	(6)	175.4 ± 11.9	***
Monomethyl-citalopram	CH_3	H	52.2 ± 4.6	(6)	303.1 ± 36.2	(6)	5.8 ± 0.5	***
Des-methyl-citalopram	H	H	245.4 ± 42.0	(6)	392.3 ± 37.1	(6)	1.7 ± 0.2	
(*S*)- Citalopram	CH_3	CH_3	29.8 ± 5.2	(8)	7924.8 ± 659.8	(8)	320.1 ± 52.2	***
(*S*)-monomethyl-citalopram	CH_3	H	70.3 ± 13.6	(6)	774.1 ± 93.4	(6)	11.8 ± 0.9	

Table 2. Impact of hSERT S438T on inhibition constants for competition of [^{3}H]MADAM binding for SERT inhibitors

The K_i-values for SERT inhibitors were determined at hSERT WT and hSERT S438T in a [^{3}H]MADAM competition binding assay as described in "Experimental Procedures". Data represents mean ± S.E.M. from at least 3 independent experiments (number of replicates given in parantheses) each performed in dublicate. The number of methyl-groups on the aminopropyl chain of the inhibitors is indicated. **, $P < 0.01$ (Student's t test).

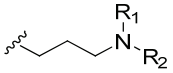
Compound			hSERT WT K_i (nM)		hSERT S438T K_i (nM)		Affinity change K_i(S438T)/K_i(WT)	
	R_1	R_2						
Imipramine	CH_3	CH_3	8.9 ± 1.1	(6)	1875.5 ± 572.4	(4)	282.6 ± 142.7	**
Desipramine	CH_3	H	237.7 ± 55.6	(5)	2038.0 ± 292.8	(4)	11.0 ± 3.9	
Clomipramine	CH_3	CH_3	0.6 ± 0.2	(7)	110.9 ± 52.5	(4)	107.2 ± 36.3	**
Monomethyl-clomipramine	CH_3	H	11.2 ± 2.7	(7)	114.3 ± 66.3	(3)	8.9 ± 1.1	
Amitriptyline	CH_3	CH_3	24.8 ± 7.1	(7)	498.2 ± 113.2	(3)	19.7 ± 6.5	**
Nortriptyline	CH_3	H	156.5 ± 37.8	(5)	289.9 ± 169.0	(4)	2.6 ± 1.7	
Citalopram	CH_3	CH_3	7.4 ± 1.9	(8)	9699.0 ± 1288.6	(4)	2148.3 ± 1004.0	**
Monomethyl-citalopram	CH_3	H	19.8 ± 2.5	(5)	155.7 ± 34.0	(4)	7.9 ± 1.0	

Figure 1

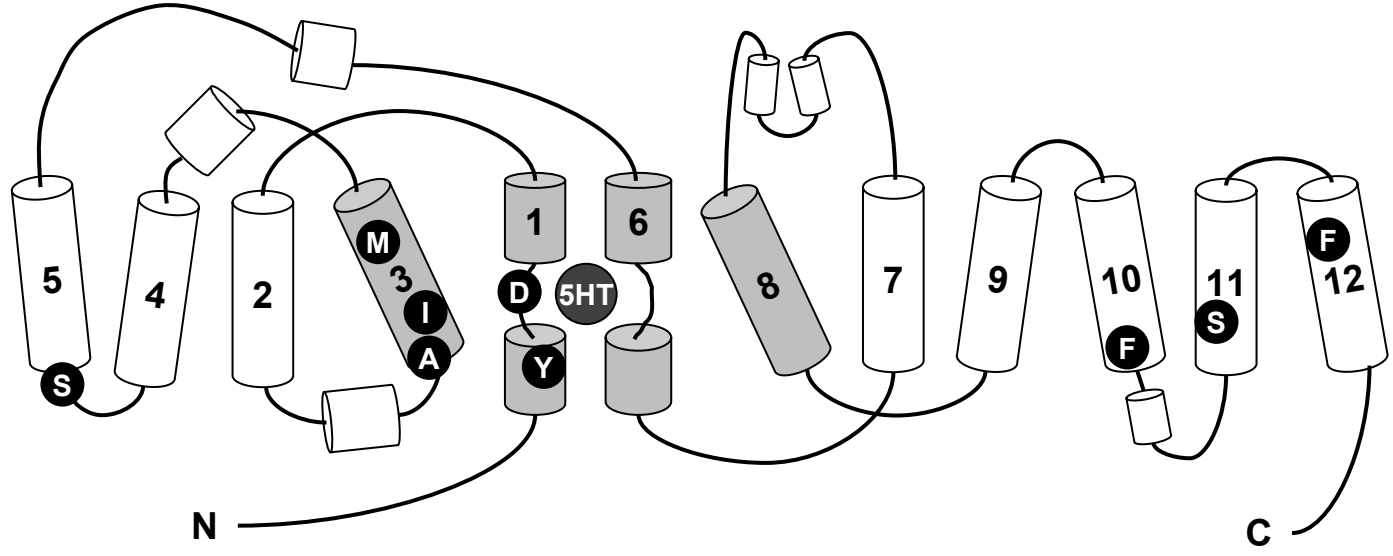


Figure 2

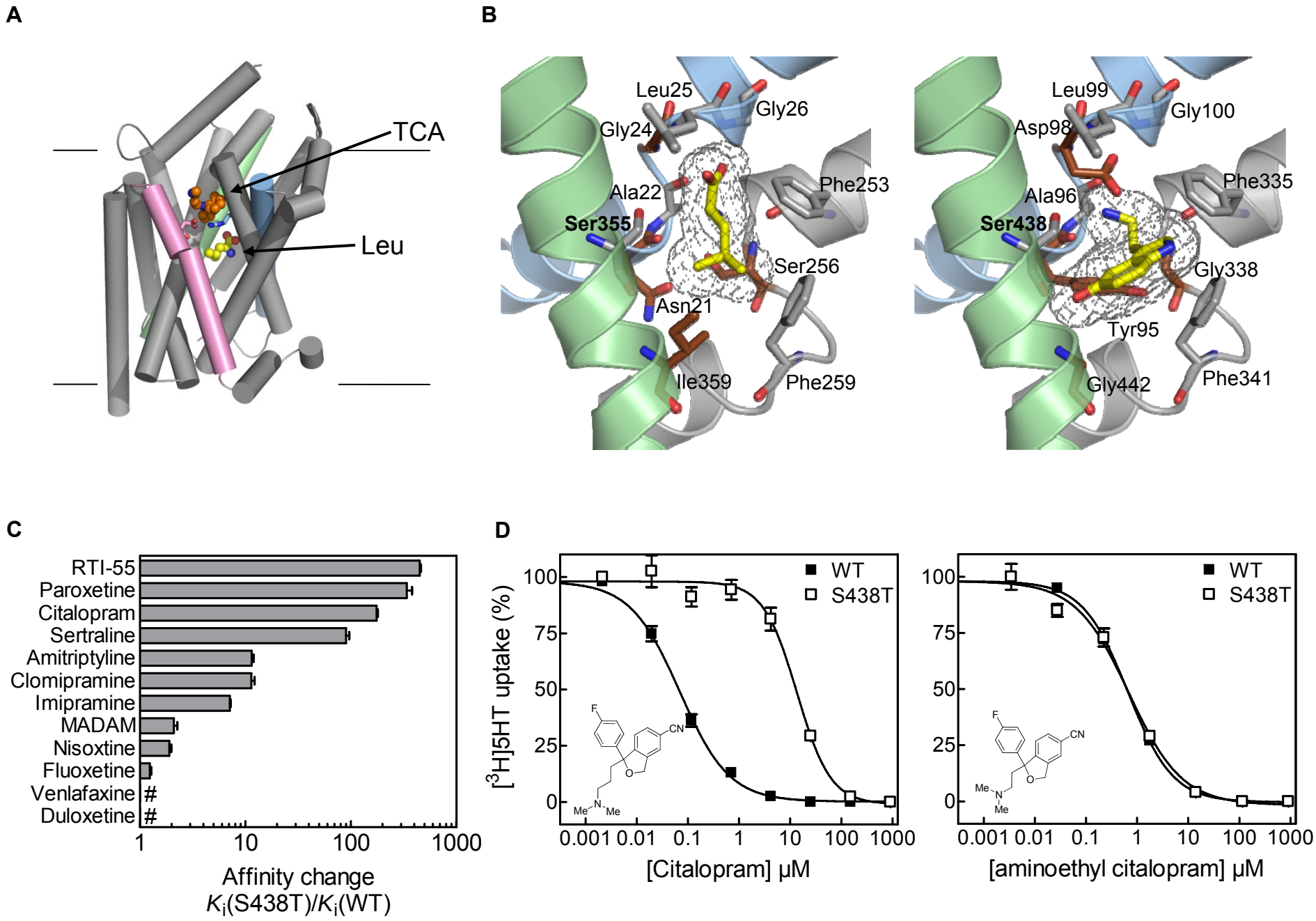

A
TCA
Leu
B
Leu25
Gly26
Gly24
Ala22
Ser355
Phe253
Ser256
Asn21
Ile359
Phe259
Leu99
Gly100
Asp98
Ala96
Ser438
Phe335
Gly338
Tyr95
Gly442
Phe341
C
RTI-55
Paroxetine
Citalopram
Sertraline
Amitriptyline
Clomipramine
Imipramine
MADAM
Nisoxtine
Fluoxetine
Venlafaxine #
Duloxetine #
1
10
100
1000
Affinity change
K_i(S438T)/K_i(WT)
D
[^{3}H]5HT uptake (%)
WT
S438T
[Citalopram] µM
[aminoethyl citalopram] µM

Figure 3

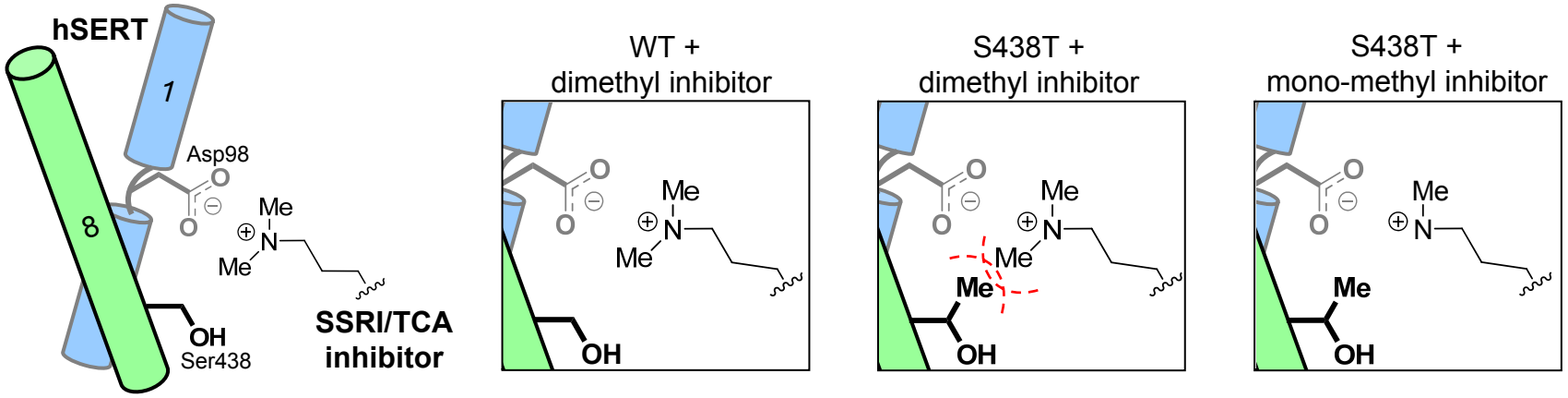

hSERT
1
8
Asp98
O
O
Me
N
Me
OH
Ser438
SSRI/TCA
inhibitor
WT +
dimethyl inhibitor
S438T +
dimethyl inhibitor
S438T +
mono-methyl inhibitor

Figure 4

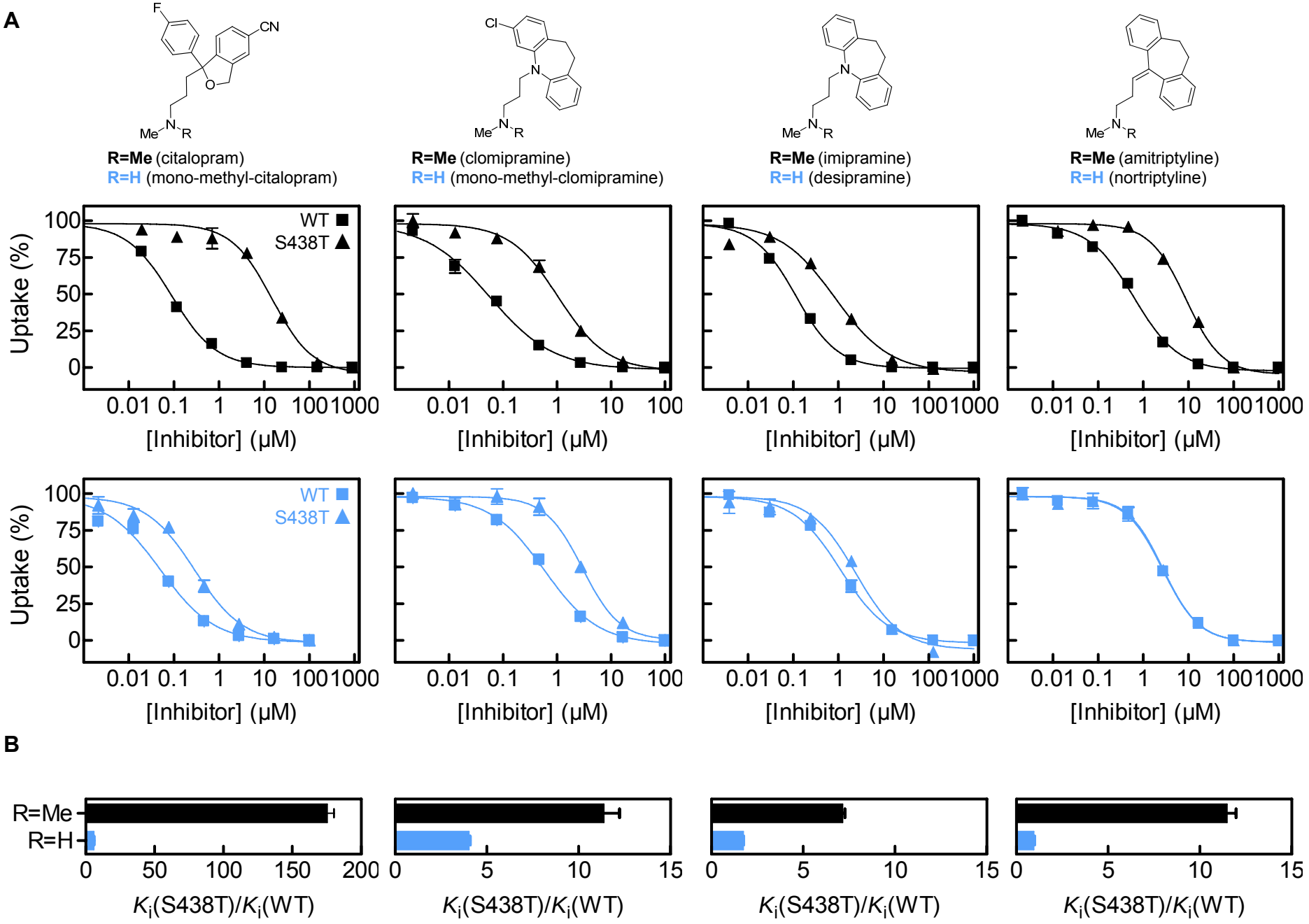
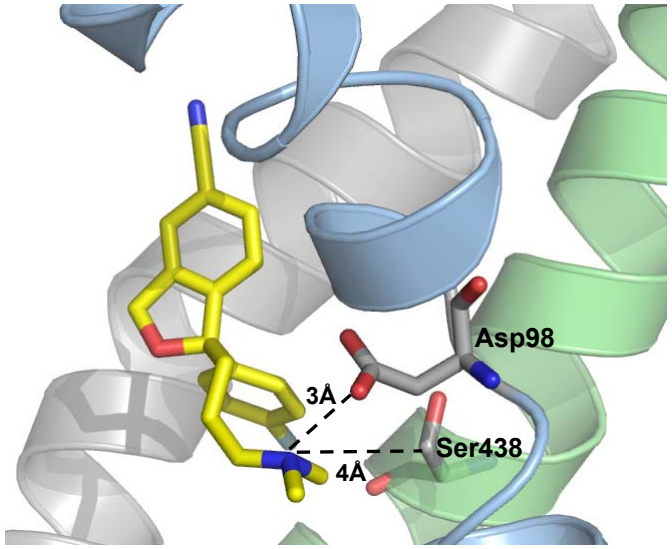

A
F
CN
O
Me
N
R
R=Me (citalopram)
R=H (mono-methyl-citalopram)
Cl
N
R=Me (clomipramine)
R=H (mono-methyl-clomipramine)
R=Me (imipramine)
R=H (desipramine)
R=Me (amitriptyline)
R=H (nortriptyline)
Uptake (%)
WT
S438T
[Inhibitor] (μM)
B
R=Me
R=H
K_i(S438T)/K_i(WT)

Figure 5

A



B

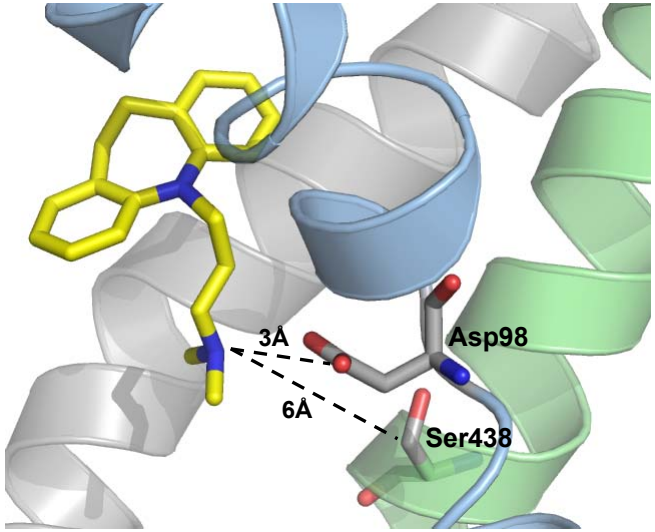
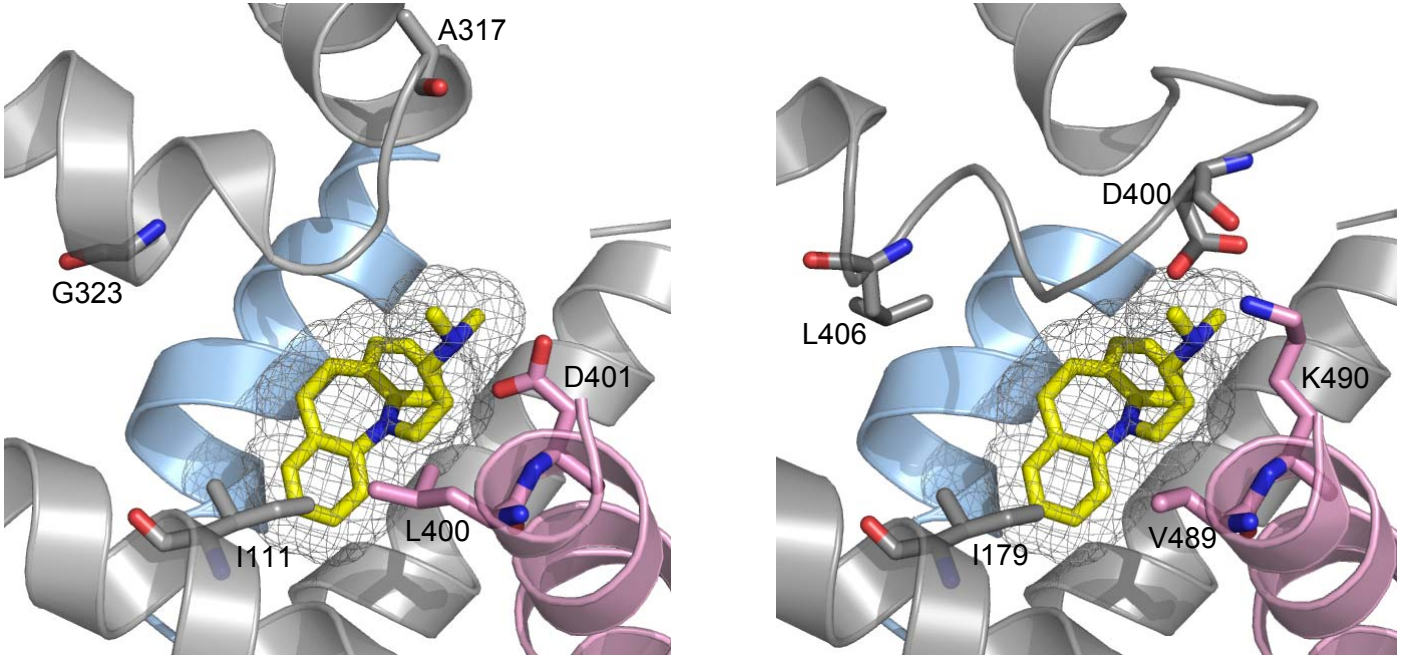


Figure 6

A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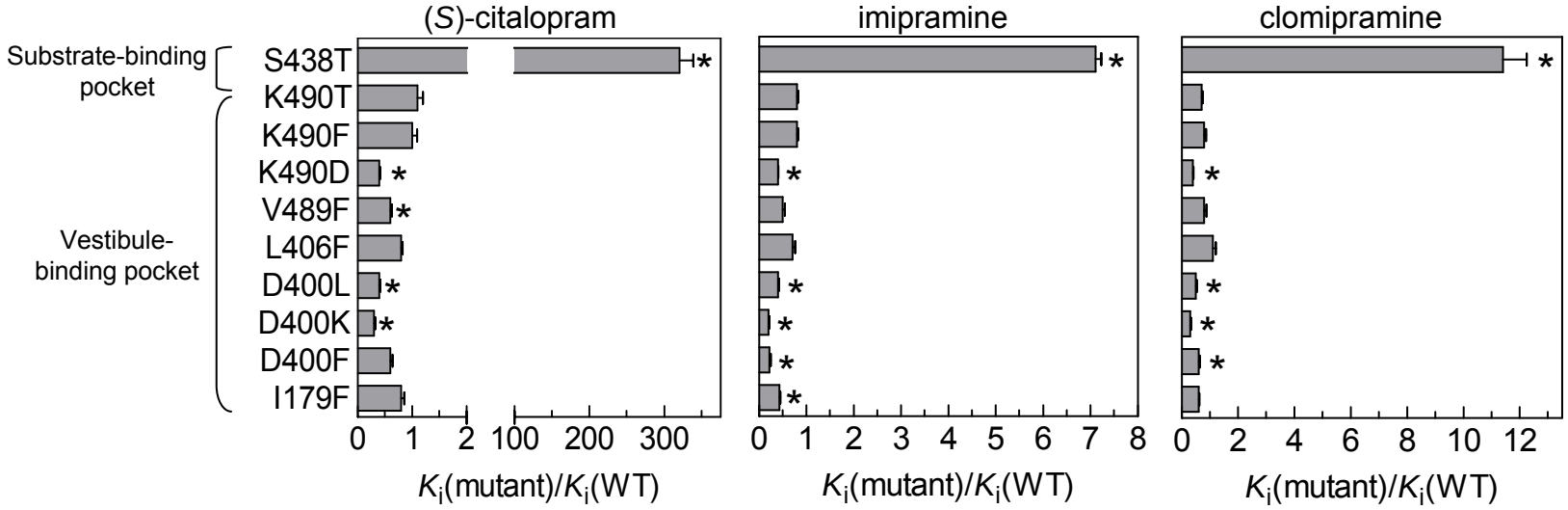
L400
I111
D400
L406
K490
V489
I179

B


(S)-citalopram
imipramine
clomipramine
Substrate-binding pocket
Vestibule-binding pocket
S438T
K490T
K490F
K490D
V489F
L406F
D400L
D400K
D400F
I179F
0 1 2 100 200 300
0 1 2 3 4 5 6 7 8
0 2 4 6 8 10 12
Ki(mutant)/Ki(WT)
Ki(mutant)/Ki(WT)
Ki(mutant)/Ki(WT)